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Research Article **Reproductive biology**

Pelvic organ prolapse (POP) is a common, debilitating disorder affecting millions of women. Uterosacral ligaments (USLs) are the main supportive structures of the uterus and vagina and are often attenuated in women with POP. Although the mechanical strength of USLs is known to be dependent on collagen synthesis and catabolism and the degradation protein MMP2 has been implicated in POP, the molecular mechanisms involved in the development of POP are currently unknown. Homeobox (*HOX*) genes are transcriptional regulators that orchestrate embryonic development of the urogenital tract. We demonstrated here that *HOXA11* was essential for organogenesis of the USL by showing that USLs were absent in *Hoxa11*-null mice. We compared expression of *HOXA11*, *collagen type I*, *collagen type III*, *MMP2*, and *MMP9* in USLs of women with and without POP. Expression of *HOXA11* and both collagens was dramatically decreased while *MMP2* was increased in women with POP. Constitutive expression of *Hoxa11* in murine fibroblasts resulted in significantly increased expression of *collagen type III* and decreased expression of *MMP2*. These results identified *HOXA11* as an essential gene for the development of the USL and suggested that women with POP might have weakened connective tissue due to changes in a signaling pathway involving *HOXA11*, *collagen type III*, and *MMP2*.

HOXA11 is critical for development and maintenance of uterosacral ligaments and deficient in pelvic prolapse

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Pelvic organ prolapse (POP) is a common, debilitating disorder affecting millions of women. Uterosacral ligaments (USLs) are the main supportive structures of the uterus and vagina and are often attenuated in women with POP. Although the mechanical strength of USLs is known to be dependent on collagen synthesis and catabolism and the degradation protein MMP2 has been implicated in POP, the molecular mechanisms involved in the development of POP are currently unknown. Homeobox (*HOX*) genes are transcriptional regulators that orchestrate embryonic development of the urogenital tract. We demonstrated here that *HOXA11* was essential for organogenesis of the USL by showing that USLs were absent in *Hoxa11*-null mice. We compared expression of *HOXA11*, *collagen type I*, *collagen type III*, *MMP2*, and *MMP9* in USLs of women with and without POP. Expression of *HOXA11* and both collagens was dramatically decreased while *MMP2* was increased in women with POP. Constitutive expression of *Hoxa11* in murine fibroblasts resulted in significantly increased expression of *collagen type III* and decreased expression of *MMP2*. These results identified *HOXA11* as an essential gene for the development of the USL and suggested that women with POP might have weakened connective tissue due to changes in a signaling pathway involving *HOXA11*, *collagen type III*, and *MMP2*.

Introduction

Pelvic organ prolapse (POP) is a common, costly, and debilitating disorder that negatively impacts the quality of life in many women. It entails the downward descent of the pelvic organs (uterus, vagina, bladder, and rectum) in women, causing symptoms including urinary and fecal incontinence, pelvic pain, and sexual dysfunction (1). The prevalence of POP has been estimated at 30%–50% of the population and increases with advancing age (2). It has been cited as one of the most common diagnoses for performing a hysterectomy, and over 300,000 procedures for POP are performed yearly in the United States (3, 4). Unfortunately, the recurrence rate for POP is very high, and approximately 30% of women undergoing surgical repair of POP will require reoperation for recurrent prolapse (4). Direct costs of this surgery exceed 1 billion dollars annually in the United States, and it is estimated that the rate of women seeking treatment for POP will double over the next 30 years as the elderly population rapidly expands (5, 6).

While increasing age, parity, menopause, and BMI have been consistently implicated as risk factors for the development of POP, the molecular mechanisms responsible for the maintenance of the pelvic organ support structures are poorly characterized (1, 7–9). It has been proposed that alterations in remodeling of the pelvic floor after childbirth trauma, compounded by the risk factors cited above, lead to gradual weakening of the pelvic support system. In women with POP, biochemical analyses of the supportive structures of the pelvic floor (endopelvic fascia and uterosacral ligaments [USLs]) have demonstrated alterations in ECM metabolism, including changes in the content and quality of collagen and expression of MMPs (10–14). Investigation of the molecular basis of POP may aid in identifying women at risk for developing prolapse and improve prevention and treatment strategies for this problem.

Homeobox (*HOX*) genes are evolutionarily conserved genes encoding transcription factors that regulate mammalian embryonic growth and development of the urogenital tract. The *HOXA* cluster genes mediate segmental differentiation of the paramesonephric duct into morphologically distinct organs of the female reproductive tract. Their collinear expression occurs in a temporal, spatial, and tissue-specific fashion (15). Specifically, *HOXA11* is responsible for the development of the lower uterine segment and cervix. In both humans and mice, this gene has been shown to persist in the adult reproductive tract and is thought to play an important role in maintaining plasticity of the uterus during times of hormonal and structural changes that occur during the menstrual cycle and in pregnancy (15–17).

Currently, it is not known whether *HOXA* genes are involved in the development of the pelvic support system. The paired USLs are key structures in this system and consist of smooth muscle and collagen. They originate at the cervix and course posterolaterally to their distal attachments at the sacrum, providing support to the uterus and upper vagina (18–20). Based on their origin of insertion at the cervix, we hypothesized that USL development and regulation involves *HOXA11*. In women with POP, the USL is often attenuated; therefore, we further hypothesized that expression of *HOXA11* would be altered in women with POP and sought to evaluate the role of this transcription factor on the homeostasis of the extracellular matrix in the USL. Here we determined that *HOXA11* was expressed in the USLs of mice and humans and that targeted deletion of Hoxa11 in mice resulted in loss of the USLs. Further-

Nonstandard abbreviations used: HOX, homeobox; PBST, PBS with 0.1% Tween-20; POP, pelvic organ prolapse; USL, uterosacral ligament.

Conflict of interest: The authors have declared that no conflict of interest exists.

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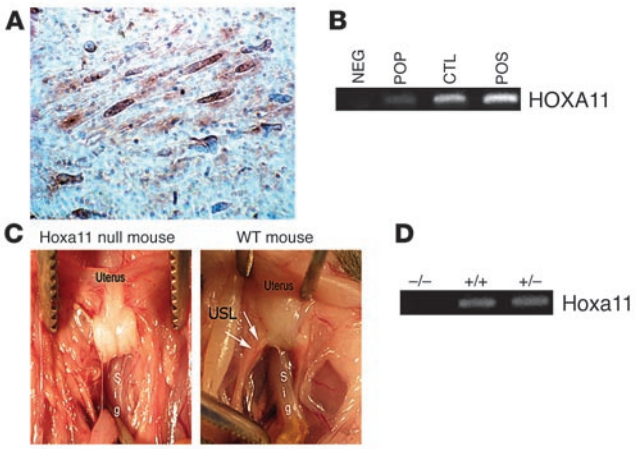

Figure 1
Evaluation of *HOXA11* expression in human and murine USLs. (**A**) Intranuclear expression of *HOXA11*demonstrated in human USLs. (**B**) PCR products confirming *HOXA11* expression in human USLs. NEG, negative control; POP, RT-PCR using RNA obtained from a subject with POP; CTL, RT-PCR using material from a control subject; POS, positive control. (**C**) Comparison of pelvic anatomy between *Hoxa11*-null and wild-type mice. Note the absence of the USLs and increased mobility of the uterus, allowing visualization of the posterior vagina, in the *Hoxa11*-null mouse compared with the definitive USLs in the wild-type mouse, which stabilizes the uterus and vagina. Arrows indicate USLs arising from the cervix. Sig, sigmoid colon. (**D**) RT-PCR results showing expression of Hoxa11 in the USLs of wild-type and Hoxa11 heterozygous mice. Paracervical tissue was used for RT-PCR in Hoxa11-null mice.

more, we determined that women with POP had altered *HOXA11* expression compared with women with normal support. *HOXA11* regulated interstitial collagen type III as well as MMP2, which degrades fibrillar collagen, a major component of USL strength.

Results

HOXA11 is expressed in the USLs of humans and mice and is necessary for their development. In humans and rodents, the uterus and upper vagina are supported by the USLs, which arise from the posterolateral aspect of the cervix (19–21). We investigated the role of *HOXA11* on the development of the USLs by evaluating the expression of *HOXA11* in these support structures in both species.

We evaluated 5 USL specimens, taken from women undergoing benign gynecological surgery, for the expression of *HOXA11* using immunohistochemistry and real-time PCR. Expression of *HOXA11* in the USL specimens was demonstrated in the nuclei, the expected subcellular location of transcription factors. Expression was also demonstrated using real-time PCR, with confirmation of products seen on an agarose gel (Figure 1, A and B).

Similar findings were present in mice. The USLs of 4 wild-type C57 Black mice were harvested under a dissecting microscope. USL size averaged 4 mm in length. Expression of *Hoxa11* was also demonstrated in these ligaments using real-time PCR. To further investigate the relationship of *Hoxa11* and USL development, we evaluated the anatomy of these ligaments in 4 *Hoxa11*-null mice and 2 heterozygous mice compared with that of the wild-type mice. Mice from 3 different litters were evaluated to ensure consistency of findings. After careful dissection, the USLs were clearly identified in all wild-type and heterozygous mice. There were no anatomical differences observed between the heterozygous and wild-type mice. Conversely, the USLs were absent in all *Hoxa11*-null mice. The USLs of wild-type and heterozygous mice and the parauterine tissue (peritoneum) of *Hoxa11*-null mice were removed, and *Hoxa11* mRNA expression was evaluated. *Hoxa11* expression was demonstrated in the USLs of wild-type and heterozygous mice, and, as expected, the parauterine tissue of the *Hoxa11*-null mice did not express *Hoxa11* mRNA (Figure 1, C and D). There was no significant difference in *Hoxa11* expression between the heterozygous and wild-type mice.

HOXA11 is decreased in women with POP. To determine whether a relationship between *HOXA11* expression and the integrity of human USLs exists, we used real-time PCR and immunohistochemistry to characterize the expression of *HOXA11* in surgical USL specimens of women with and without POP.

We evaluated 18 women with POP and 10 women with normal pelvic support in this study. The cohort in this study population was predominantly white (85.8%). The remainder of the participants consisted of women of African American (7.1%) and Hispanic (7.1%) ancestry. There were no differences in age, menopausal status, and BMI between the 2 groups (Table 1). None of the postmenopausal women in either group were taking hormone replacement therapy. In the POP group, 9 women had stage II POP and 9 women had stage III POP. All women in this group underwent surgery for symptomatic prolapse. All of the women with POP had urinary incontinence; none of the controls had this condition.

Histological evaluation with trichrome staining of all specimens confirmed that biopsied tissue was indeed USL. Alterations in the collagen architecture seen in our population with POP included more loosely organized collagen compared with women with normal pelvic support (Figure 2).

Immunohistochemistry demonstrated expression of *HOXA11* in the USLs from both groups, although the positive immunoreactivity of intranuclear staining was much less pronounced in the POP group compared with controls (Figure 3A). Comparison of the relative expression of *HOXA11* mRNA between women with and without POP showed similar results. Expression of *HOXA11* was 75-fold lower in women with POP compared with controls ($P < 0.01$) despite similarities in age, parity, menopausal status, and BMI between the groups (Figure 3B).

Expression of ECM proteins are also altered in the USLs of women with POP. Alterations in the ECM in the USLs in women with POP have been previously reported and are thought to play a role in the

Table 1
Characteristics of enrolled women with and without prolapse

	Control ($n = 10$)	POP ($n = 18$)	*P*
Age (yr)	46.5 ± 14.8	54 ± 11.5	0.15
Parity	1.4 ± 1.8	2.7 ± 1.6	0.05
Menopause	40%	38.9%	0.64
BMI (kg/m^2)	24.8 ± 6.7	26.0 ± 8.0	0.65

Values are mean ± SEM.

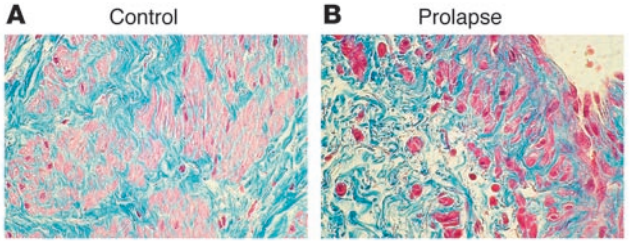

Figure 2
Trichrome stain of human USL tissue. Original magnification, ×60. (**A**) Sample obtained from a premenopausal woman with normal pelvic support. (**B**) Sample obtained from a premenopausal woman with stage III POP. Note the altered appearance of collagen formation.

development of this condition (11–14). Semiquantitative evaluations have previously been used to evaluate these relationships. Based on previous reports and our observed differences in the histological appearances of the USL specimens between our groups, we compared the mRNA expression of these genes in the USLs of women with and without POP using real-time PCR. We found that the expression of both collagen type I and collagen type III was significantly reduced in the USLs of women with POP compared with controls. We observed 7- and 17-fold reductions in expression of collagen type I and collagen type III, respectively (Table 2).

In addition to altered collagen in the pelvic connective tissues, increased MMP activity has been implicated in the development of POP and is thought to be involved in the attenuation of the USL (12–14). Evaluation of the expression of MMP2 and MMP9 revealed that expression of these catabolic enzymes was increased by 2- and 3.2-fold for MMP2 and MMP9, respectively, in women with POP. However, the change in expression for MMP9 was not statistically significant (Table 2).

Hoxa11 regulates collagen and MMP expression. *HOXA11* encodes for transcription factors that regulate downstream gene activity and orchestrate organogenesis of the lower uterus and cervix, which are composed of smooth muscle and collagen (15). After observing lower expression of *HOXA11* and collagen accompanied by higher expression of MMPs in the USLs of patients with POP compared with controls, we investigated the role of *HOXA11* in the regulation of interstitial collagens and MMPs involved in their catabolism. We explored this concept in vitro by constitutively expressing *Hoxa11* in NIH 3T3 murine embryonic fibroblasts. After transient transfection with pTriEx-4/*Hoxa11* (see Methods), we measured the resultant expression of collagen types I and III, MMP2, and MMP9 using real-time PCR. Empty vector (without the Hoxa11 insert) was used as a control, and transfection efficiency was confirmed by cotransfection with a vector containing the reporter gene GFP.

Figure 3
HOXA11 mRNA and protein expression in the USLs of women with and without POP. (**A**) *HOXA11* expression in USLs shown by immunohistochemistry. Original magnification, ×60. Note the strong *HOXA11* expression in the USLs of a premenopausal subject with normal pelvic support compared with the weak expression of *HOXA11* in the USLs of a subject with POP. (**B**) Comparison of mRNA expression of *HOXA11* in USLs in women with and without POP. Expression of *HOXA11* was dramatically reduced in women with POP versus controls ($P < 0.01$).

Approximately 30%–40% of cells were GFP positive. *Hoxa11* expression levels were compared between cells transiently transfected with pTriEx-4/*Hoxa11* and empty vector using real-time PCR. *Hoxa11* expression was over 1,000-fold higher in cells transfected with the *Hoxa11* vector compared with controls. Expression of collagen type III was significantly increased by 2.8-fold in the NIH 3T3 cells after transient transfection with the *Hoxa11* vector compared with cells treated with an empty vector. In contrast, MMP2 expression significantly decreased with constitutive expression of Hoxa11 compared with controls. Expression of collagen type I and MMP9 was not significantly affected by overexpression of *Hoxa11* (Table 3).

Discussion

Although much is known about the growth and differentiation of the reproductive tract, the molecular pathways involved in the development and maintenance of the USL are not well understood. The present study provides insight on the role of *HOXA11* in the embryological development of the USLs, the key supporting ligaments of the uterus and vagina. To our knowledge, this is the first gene identified as necessary for USL development in any species.

The pathogenesis leading to attenuation of the USL in patients with POP is unclear. Cadaveric studies have shown that USLs are incredibly strong structures for their size, capable of supporting more than 17 kg of weight before failure (22). However, the resiliency of USLs in women with POP has previously been shown to be reduced by more than 4-fold compared with women with normal support, suggesting intrinsic deficiencies in the quality of the USLs in these women (23).

The mechanical strength of the supportive pelvic structures is dependent on the quantity and quality of the collagen fibers. Tissue remodeling is a continuous process, and a delicate balance between matrix synthesis and breakdown is necessary to maintain the integrity of the tissue. Degradation of collagens I and III is

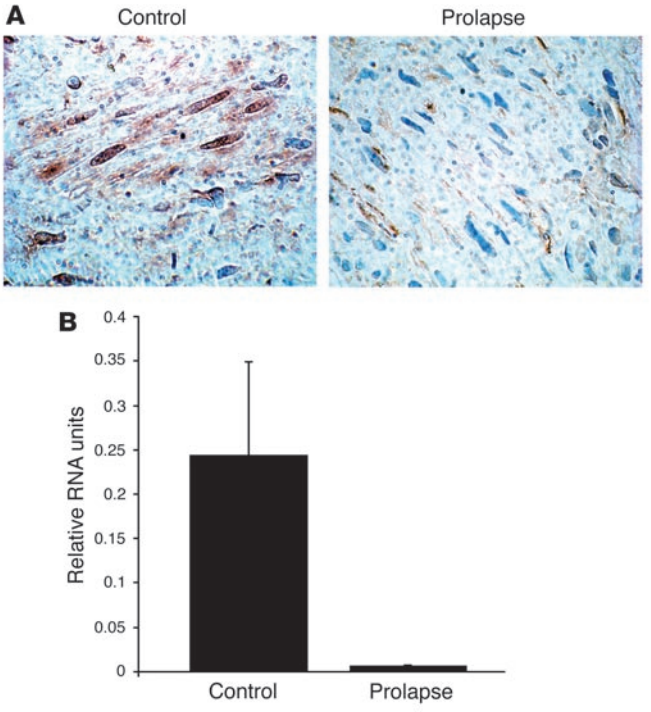

Table 2
mRNA expression of collagens and MMPs in USLs from women with POP

	Change	Fold	*P*
Collagen type I	↓	7.3	0.03
Collagen type III	↓	17.0	<0.01
MMP2	↑	2.0	0.04
MMP9	↑	3.2	0.23

↓, decrease; ↑, increase. Fold change relative to control subjects is shown.

dependent on the activity of the interstitial MMPs, which ultimately receive input from MMP2 and MMP9 (24–26).

Previous studies have examined the relationship regarding the quality and quantity of collagen in USLs in women with POP. Several immunohistochemical studies have shown a higher percentage of immunohistochemically positive areas along with increased density of collagen and decreased cellularity in patients with POP (10, 27). These findings are in contrast to our results which showed decreased procollagen in our subjects with POP. This may be explained by the findings of Suzme et al. and Lang et al., who describe increased diameter of collagen fibrils in patients with POP and decreased hydroxyproline, which is responsible for the tightly bound crosslink between fibrils (14, 28). These findings, coupled with our present results, suggest that fewer fibers of increased diameter size are present in USLs of patients with POP.

The decrease in collagen expression may also reflect the end result of sustained increases in MMP2 activity. We show here that proMMP2 mRNA increased 2-fold in our subjects with POP. This is consistent with findings from other authors who reported that pro- and activated MMP2 proteins are significantly increased in USLs of women with POP (12, 13). MMP2 is capable of digesting fibrillar collagen to completion and is a potent activator of MMP9 (24, 26). Our present results suggest that MMP2 plays a central role in collagen reorganization and may be a critical factor in USL attenuation associated with POP.

We found significant decreases in *HOXA11* and collagen expression, accompanied by increased expression of MMP2, in the USLs of subjects with POP. Our in vitro data clarified this association and offer insight into what we believe to be a previously unknown pathway of ECM metabolism in these support structures. Expression of *Hoxa11* increased procollagen type III expression and decreased MMP2 expression in murine fibroblasts, favoring collagen synthesis over catabolism.

Due to the cross-sectional design of this study, it is not possible to determine whether women with POP have weakened pelvic connective tissue as a result of changes in a signaling pathway involving *HOXA11* regulation of collagen type III and MMP2 or if these findings are the result of POP. However, our in vivo findings suggest that POP is associated with decreased HOXA11 and collagen type III expression and increased MMP2 expression, and our in vitro findings elucidate the regulation of collagen type III and MMP2 by *HOXA11*.

These observations extend our understanding of molecular mechanisms that regulate the maintenance of a critical ligament of the pelvic support system, the USL. Deficient *HOXA11* signaling may limit functional development or repair of the USL after trauma in susceptible women and contribute to alterations in the biomechanical strength of the USL, leading to uterovaginal prolapse. Additionally, alterations in *HOX* genes would be expected to produce a variety of defects in the expression of downstream target genes. Understanding both the regulation of *HOXA11* and its regulation of downstream signaling pathways involved in the homeostasis of the USL may offer new therapeutic strategies for women with POP and improve the quality of life for those affected by this condition.

Methods

Animals. Experiments using mice were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with approval of the Yale Animal Care and Use Committee. Nulliparous, wild-type C57 Black mice and C57 Black mice with a targeted disruption of *Hoxa11* were allowed to reach reproductive maturity and then sacrificed via cervical dislocation (*Hoxa11*-null mice were a generous gift from S.S. Potter, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA; ref. 29). Dissection of the pelvic organs was promptly performed under a dissecting microscope and documented with a high-resolution camera. The USLs of 4 wild-type and 2 heterozygous mice and parauterine tissue of 4 *Hoxa11*-null mice were removed and placed in RNA later and stored at –80°C until extraction of RNA was performed.

Acquisition of human tissue. All experiments were performed with approval of the Yale Human Investigation Committee. Specimens were collected between September 2005 and July 2007 from women undergoing hysterectomy or laparoscopic uterine nerve ablation at our institution. Informed consent was obtained from all patients. Prior to the surgery, a pelvic examination was performed to evaluate for the presence of POP. Uterovaginal prolapse was graded according to the POP quantification system advocated by the International Continence Society (30). Women with stage II POP or greater were assigned to the POP group. Data regarding age, menopausal status, and parity were recorded. All patients were screened for urinary incontinence (urge and stress), and all patients with POP underwent preoperative multichannel urodynamic testing (Medtronic).

At the time of surgery, 3- to 5-mm samples of the USLs were taken from the proximal ligament at its insertion into the cervix, where the ligament is consistently identifiable. Specimens were divided in half. One section was promptly placed in RNA*later* (Ambion Inc.) and stored at –80°C until extraction of RNA was performed. The other half was placed in 10% formalin for immunohistochemistry.

Immunohistochemistry. Formalin-fixed specimens were embedded in paraffin, sectioned (5 μm thickness), and fixed to glass slides. Specimens were deparaffinized in serial xylene and ethanol washes and permeabilized in cold 95% ethanol for 10 minutes. After rinsing in deionized water for 10 minutes, the antigens were retrieved by steaming the slides in a 0.01 M sodium citrate buffer for 20 minutes followed by a 20-minute cooling period. Slides were rinsed in PBS for 3 minutes, followed by PBS with 0.1% Tween-20 (PBST). Endogenous peroxidase was quenched using a 3% hydrogen peroxide solution for 5 minutes, and slides were then washed in

Table 3
mRNA expression of collagens and MMPs following overexpression of *Hoxa11* in murine fibroblasts

	Change	Fold	*P*
Collagen type I	↑	1.26	0.17
Collagen type III	↑	2.8	<0.01
MMP2	↓	1.8	0.03
MMP9	↑	1.29	0.94

↓, decrease; ↑, increase. Fold change relative to baseline is shown.

Table 4
Primer sequences used for real-time PCR reactions evaluating expression of Hoxa11, procollagen type I and III, β-actin, proMMP2, and proMMP9 in the murine cell line NIH/3T3

Primer	Sequence
Hoxa11 forward	5′-CAATCTGGCCCACTGCTAC-3′
Hoxa11 reverse	5′-GTTGCAGACGCTTCTCTTT-3′
Procollagen type Ia1 forward	5′-GAGCGGAGAGTACTGGATCG-3′
Procollagen type Ia1 reverse	5′-GTTCGGGCTGATGTACCAGT-3′
Procollagen type IIIA1 forward	5′-AGGCTGAAGGAAACAGCAAA-3′
Procollagen type IIIa1 reverse	5′-TAGTCTCATTGCCTTGCGTG-3′
ProMMP-2 forward	5′-CGGTTTATTTGGCGGAC-3′
ProMMP-2 reverse	5′-TGCGATGAGCTTAGGG-3′
ProMMP-9 forward	5′-TGTACGGACCCGAAGC-3′
ProMMP-9 reverse	5′-CCGTCCTTATCGTAGTCAG-3′
β-Actin forward	5′-AGAGGGAAATCGTGCGTGAC-3′
β-Actin reverse	5′-CAATAGTGATGACCTGGCCGT-3′

PBST for 3 minutes. Sections were circumscribed with a hydrophobic pen, and normal goat serum (1.5%) was placed on specimens and incubated for 1 hour at room temperature to block nonspecific binding. The slides were then incubated overnight at 4°C with *HOXA11* antibody (made in rabbit, catalog no. ab-28699; Abcam Inc.) at 1:500 concentration. Following the overnight incubation, slides were washed in 1× PBST, incubated in ABC Elite (Vector Laboratories), washed in 1× PBST, and incubated in 3,3-diaminobenzidine (Vector Laboratories) for 40 seconds. Slides receiving ABC Elite with rabbit IgG as the primary antibody were used as negative controls. To achieve counterstaining, slides were exposed with filtered hematoxylin for 20 seconds each and rinsed with deionized water. Rehydration was then performed using 3-minute ethanol and xylene washes. Slides were mounted with permount. Photographs of the slides were taken at ×20 magnification under the same lighting conditions using a Kodak DC290 Zoom digital camera (Eastman Kodak). To ensure specificity of the *HOXA11* antibody, we performed immunohistochemistry on paracervical tissue obtained from *Hoxa11*-null mice. No immunostaining was observed, indicating lack of cross-reactivity of anti-*Hoxa11* with other related *HOX* proteins.

RNA extraction. RNA specimens from mouse and human tissues were thawed on ice and placed in 1 ml TRIzol per 100 mg tissue (Invitrogen). Tissue was homogenized on ice, and total RNA was isolated using methodology described by the manufacturer. The RNA from transfected murine cell cultures were also extracted using TRIzol. The OD of the RNA was measured using SmartSpex 300 (BioRad Laboratories Inc.) at 260 nm to determine the concentration. We reverse-transcribed 1 μg high-purity RNA (OD 260/280 ratios, 1.8–2.0) using the Eppendorf Mastercycler (Eppendorf North America) and the BioRad iScript cDNA synthesis kit. The reaction mix was incubated for 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C.

The mRNA expression of *HOXA11*, collagen type I, procollagen type III, MMP2, and MMP9 in the USLs and murine cells was determined using RT-PCR. The PCR reaction was carried out in a MyiQ Real-Time PCR detection system (BioRad Laboratories Inc.) with the following cycling conditions: preincubation at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 62°C for 20 seconds for the human and murine *HOXA11* (61°C for human and murine collagen types I and III, MMP2, and MMP9), and extension at 72°C for 25 seconds. The melting peak of each sample was routinely determined by melting curve analysis to ascertain that only the expected products had been generated, and all specimens were run in duplicate. Negative controls were run in the absence of reverse transcriptase. The murine and human primer sequences for *HOXA11*, collagen I, collagen III, MMP2, and MMP9 are listed in Tables 4 and 5. Samples were run in duplicate, and experiments were repeated twice. The PCR products of the USLs of the wild-type, heterozygous, and *Hoxa11*-null mice were confirmed by expression on a 1.5% agarose gel electrophoresis.

Gene expression levels were standardized by calculating mRNA ratios relative to the housekeeping gene β-actin. Ct was set to ensure the curve was crossed at a linear point. The difference in the mean Ct (ΔCt) between the housekeeping gene β-actin and our genes of interest (*HOXA11*, collagen I, collagen III, MMP2, and MMP9) was determined. The formula $1/2^{\Delta Ct}$ was used to compare relative values of RNA. An unpaired Student's *t* test was performed to determine statistical differences between the 2 groups. Fold changes in mRNA expression after transfections were calculated using the formula $2^{\Delta Ct-\Delta Ct}$ assuming 100% PCR efficiency.

Constitutive expression of Hoxa11 in murine 3T3 cells. NIH 3T3 mouse fibroblast cells were cultured at 37°C in 90% DMEM with 4mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, and 10% calf serum. Cells were plated at 1×10^5 in 6-well plates and grown over 24 hours to an approximate 50%–70% confluence. Duplicate wells were transfected with a pTriEX-4 vector (Novagen), with a cytomegalovirus promoter constitutively expressing Hoxa11 or with an empty vector, and TransIT- LT1 (Mirus Bio Corporation), a high-efficiency, low toxicity transfection reagent, according to the manufacturer's protocol. The vector with the *Hoxa11* insert was a generous gift from the Gunter Wagner Laboratory of Yale University. A cotransfection with a vector containing the reporter gene GFP was performed in the third well with each of the other vectors to evaluate transfection efficiency. At 24 hours after transfection, total RNA was isolated and processed, and RT-PCR was performed as described above to evaluate effects on expression of collagens type I and III, MMP2, and MMP9. The fold increase in Hoxa11 expression after tranfsection was measured using real-time PCR. In addition, the percent GFP-positive cells per 5 high-power fields were averaged. Both approaches were used to estimate transfection efficiency.

Statistics. Descriptive data are expressed as mean ± SEM and percentages. All experiments were performed in duplicate, and results were compared using Student's *t* test and χ^2 analyses. Statistical analysis was performed with Excel software (Microsoft Office 2003). A *P* value less than 0.05 was considered significant.

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Table 5
Primer sequences used for real-time PCR reactions evaluating expression of HOXA11, procollagen type I and III, and β-actin in USL specimens

Primer	Sequence
HOXA11 forward	5′-GTACTTACTACGTCTCGGGTCCAG-3′
HOXA11 reverse	5′-AGTCTCTGTGCACGAGCTCCT-3′
Procollagen type 1A1 forward	5′-AGGGCCAAGACGAAGACATC-3′
Procollagen type 1A1 reverse	5′-AGATCACGTCATCGCACAACA-3′
Procollagen type 3A1 forward	5′-TGGCTACTTCTCGCTCTGCTT-3′
Procollagen type 3A1 reverse	5′-CGGATCCTGAGTCACAGACACA-3′
ProMMP-2 forward	5′-CGGCCGCAGTGACGGAAA-3′
ProMMP-2 reverse	5′-CATCCTGGGACAGACGGAAG-3′
MMP-9 forward	5′-GACGCAGACATACGTCATCCAGTTT-3′
MMP-9 reverse	5′-GCCGCGCCATCTGCGTT-3′
β-Actin forward	5′-CGTACCACTGGCATCGTGAT-3′
β-Actin reverse	5′-GTGTTGGCGTACAGGTCTTTG-3′

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